

# Immunohistochemistry of a prostate membrane specific protein during development and maturation of the human prostate

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## ABSTRACT

An antiserum against secretory vesicles from human seminal fluid (prostasomes) was used to study the localisation and distribution of the respective antigen(s) during prenatal development and pubertal maturation of the human prostate. The crude antiserum stained both secretory and membrane proteins in the adult prostate and other glands, such as pancreas and parotid gland. An immunoaffinity purified fraction from the antiserum selectively reacted with the apical plasma membrane of prostatic epithelium adluminal cells, recognizing a 100 kDa antigen (PMS). Even in the earliest stages of embryonic prostate specimens studied, the adluminal plasma membrane of the epithelial cells from developing glandular anlagen reacted strongly. The occurrence of PMS immunoreactivity in prostatic anlagen was directly correlated with lumen formation. As the antigen is an androgen-independently synthesised membrane protein of the prostate, it may possibly be used as a marker of cell polarity in the normal and pathologically altered prostate.

**Key words:** Prostate membrane specific protein; prostate development.

## INTRODUCTION

The human prostate is derived from the upper part of the urogenital sinus in the region into which the mesonephric and paramesonephric ducts open (Cunha, 1994). During the 7th wk of gestation, male embryos develop a colliculus seminalis in the cranial part of the urethra (Kellokumpu-Lehtinen et al. 1980). By the 9th wk, the epithelium covering the colliculus consists of a monolayer of columnar cells. Prostatic development then starts with proliferation of the mesenchyme surrounding the urogenital sinus. At the age of 10 wk, when the verumontanum has developed, histological differentiation begins close to the openings of the mesonephric ducts by outgrowth of several buds of the urethral epithelium into the surrounding mesenchyme. Between the 11th and 14th wk, some of the buds have acquired a lumen and they start to transform into tubular-acinar anlagen (Kellokumpu-Lehtinen et al. 1980). This process proceeds until birth, when in the perinatal period most

of the periurethral ducts are filled with squamous metaplastic epithelium.

In human prostatic development, different cell types sequentially appear and disappear, especially in prostatic epithelium and to a lesser degree in prostatic stroma (Aumüller et al. 1994). Cells derived from urogenital sinus epithelium, containing characteristic masses of glycogen, transform into undifferentiated squamous epithelial cells. Immediately after the formation of a lumen inside the initially solid epithelial buds sprouting into the mesenchyme, the adluminal cells differentiate into polarised columnar cells, whereas undifferentiated cells remain in the basal layer which eventually form typical triangular basal cells and neuroendocrine cells.

Whereas the neuroendocrine cells can easily be recognised immunohistochemically by their content of chromogranin and different hormones (de Mesy Jensen & di Sant'Agnese, 1994), the future secretory, adluminal cells are difficult to characterise aside from their location, in that they are nearly completely

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## EXHIBIT E

devoid of secretory material, at least during embryonic development. The present study was performed (1) to test the cell specificity of an antibody directed against prostate derived membrane particles (so-called prostasomes), (2) to trace the formation, development and maturation of secretory acinar cells and (3) establish the potential application of the antibody as a marker for prostatic differentiation, both during normal development and malignant transformation.

#### MATERIAL AND METHODS

##### Antiserum

Human prostasomes were isolated from semen samples obtained from voluntary semen donors with normal fertility parameters (courtesy of Prof. Krause, Department of Andrology, University Hospital Marburg), using the method of Ronquist & Brody (1985) with minor modifications. After semen liquefaction (~45 min), seminal fluid was separated from spermatozoa by centrifugation at 1000 g for 20 °C. The supernatant was diluted 1:5 with 30 mM Tris/HCl-buffer, pH 7.5 containing 130 mM NaCl (used as a diluent buffer in all further steps) and was ultracentrifuged for 2 h at 4 °C and 105000 g. The pellet was resuspended in 1.0–1.5 ml of the buffer and further purified on a 40 ml Sephadryl S-500 HR column. The resulting purified prostasomes were then resuspended in 1 ml of diluent buffer. Immunisation of female rabbits of the New Zealand strain was undertaken by injecting a mixture of native prostasomes (150 µg of protein) and Gerbu 100 adjuvant solution (Gerbu Biotechnik, Gaiberg, Germany) subcutaneously into the back skin. Three booster injections were given during the following 12 wk. At 10 d after the last injection, blood was collected from the ear vein of the rabbit. After clotting (4 °C, 12 h), the antiserum was obtained by centrifugation for 20 min at 1500 g. The antiserum serum was characterised by Western blotting, using freshly prepared prostasomes as antigen. Antisera against PSA (MAS 343 cf, clone 8, Seralab, Crawley Down, England) and acid phosphatase (own polyclonal antibody, Aumüller et al. 1983) were used as reference standards. Details of the production and characterization of the antiserum against prostasome-derived prostate membrane specific antigen (PMS) are to be published elsewhere (Renneberg et al. 1996).

##### Prostate specimens

Organ specimens were taken from the files of the Department of Anatomy and Cell Biology, Marburg,

and the Departments of Pathology of the University Hospitals of Marburg (courtesy of Prof. Thomas), Freiburg (courtesy of Prof. Böhm) and Homburg/Saar (courtesy of Prof. Dr Bonkhoff). Specimens had been fixed in neutral formalin during routine autopsies. The following age stages were available: fetuses from the 15, 17, 19, 20–30, 36, 39 and 40 wk of gestation, prostates from 1 newborn, 3.5-mo-old and 3, 4, 5, 14, 15 and 16-y-old boys each, and 2 17-y-old, 1 18 and 1 19-y-old males. All specimens were paraffin-wax embedded. Sections were cut at 5–6 µm thickness, mounted on chromalum-gelatin subbed glass slides and used for immunohistochemistry after removal of paraffin in xylene and ethanol.

##### Immunohistochemistry

The unlabelled antibody enzyme method (Sternberger et al. 1970) was used for the polyclonal rabbit antibodies and the indirect immunoperoxidase method for the monoclonal PSA antibody. Polyclonal antibodies against prostasomes (Renneberg et al. 1996) and acid phosphatase, respectively, were applied to deparaffinised sections in a moist chamber at room temperature for 1 h, diluted 1:100 in phosphate buffered saline (PBS). After thorough rinsing of the sections in PBS, they were incubated for 30 min with the secondary antibody (antirabbit IgG, Dianova, Hamburg, Germany, diluted 1:200 in PBS). The soluble peroxidase-antiperoxidase complex (PAP, Dianova, Hamburg, diluted 1:250 in PBS) was added to the sections after thorough rinsing and incubated for 30 min. The immunoreaction was visualised by incubation in 3,3'-diaminobenzidine (DAB, 10 µg) dissolved in 100 ml PBS containing 0.001% hydrogen peroxide (Graham & Karnovsky, 1966). Sections were thoroughly rinsed in PBS and distilled water, dehydrated and mounted in a synthetic resin. For visualisation of the immunoreaction using the monoclonal anti-PSA antibody (1:20 diluted in PBS, 1 h at room temperature), a secondary peroxidase-conjugated antimonouse IgG (Dianova, Hamburg, 1:100 in PBS, 30 min) was applied and the immunoreaction was visualised using the DAB-H<sub>2</sub>O<sub>2</sub> procedure as described. In some cases, nuclear staining with haemalum was performed. After dehydration and mounting in synthetic resin, sections were evaluated in a Zeiss photomicroscope.

For ultrastructural immunolocalization, immersion-fixed (0.1% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.3) BPH specimens were cut into 50–100 µm thick sections using a tissue chopper equipped with a

razor blade. Chopper sections were incubated with the affinity-purified anti-prostasome antiserum (1:10) and subsequently with gold-labelled (5 nm particles) anti-rabbit IgG (1:50). After thorough rinsing in PBS, chopper sections were treated with 1% aqueous osmium solution for 30 min, dehydrated in alcohol and propylene oxide and embedded in Epon. Ultrathin sections were cut at 500 nm on a Reichert ultramicrotome equipped with a diamond knife, briefly stained with uranyl acetate and lead citrate and examined in a Zeiss EM 10 electron microscope. In control incubations, the primary antibody was replaced by 1% swine serum in PBS.

## RESULTS

### *Lumen formation*

The earliest embryonic stages available were from the 15th to the 26th wk of gestation, when the epithelial buds (starting to grow out from the urethra during the 10th wk) have already considerably increased in number, and transform into tubulo-acinar anlagen (Fig. 1a-c). Incipient tubule formation is recorded from the development of a lumen within the initially solid epithelial buds (Fig. 2a). The formation of the lumen during all stages of development is accompanied by the appearance of immunoreactivity for PMS in some centrally located cells (Fig. 2a). Usually 2 or 3 cells abutting each other start elongating, moving their nuclei into the direction of the basement membrane and show a positive immunoreaction at their opposite pole. Polarisation of the cells and appearance of PMS are obviously intimately corre-

lated. Shortly after polarisation of the cells, the appearance of a lumen becomes visible. The underlying 3-5 cell layers are definitely nonreactive with the very rare exception that most basal cells residing on the basement membrane develop some transient immunoreactivity at their basal plasma membrane. The adluminal cells divide frequently, thereby increasing the diameter of the lumen and reducing the height of the stratified epithelial layer. Conversely, at the tips of the solid buds, the basally located cells divide. This results in further piercing of the epithelial buds into the surrounding mesenchymal stroma. The latter acquires a more differentiated structure at the periphery, resembling a capsule which may be responsible for the termination of epithelial budding. The process of lumen formation proceeds in the same way as already described. In embryonic specimens, immunoreactivity for PSA or acid phosphatase was never observed in adluminal PMS-immunoreactive or any other epithelial and neuroendocrine cells.

### *Metaplasia*

Shortly after birth, the larger ducts surrounding the urethra, resume proliferative activity of the basal and intermediate cells, developing a pronounced squamous metaplasia (Fig. 2b,c). Squamous metaplastic cells are removed by simple desquamation into the lumen (Fig. 2b), where they condense and form thick masses of detritus. Within the thick layers of metaplastic epithelium, lumen formation occurs, with differentiation of adluminal cells containing apical PMS immunoreactivity. Sometimes very extensive

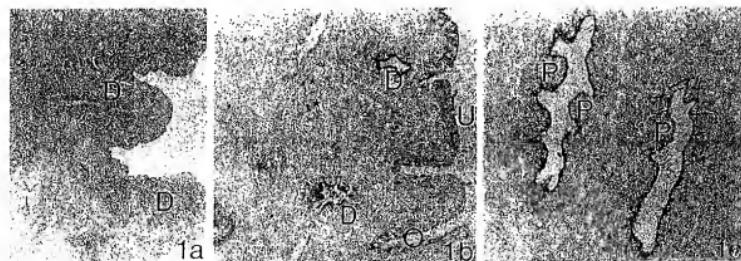


Fig. 1. Immunoreaction of the PMS antiserum in the prostate of an embryo from the 26th wk of gestation. (a) Survey of the prostatic anlagen with strongly labelled large ducts (D).  $\times 2$ . (b) Lateral perirethral budding zone. Note the irregular and moderate staining of the urethral epithelium (U). Homogeneous immunoreactivity is present only in prostatic ducts (D) somewhat distant of their urethral orifices (O). A few immunoreactive macrophages (asterisks) are seen in the stroma.  $\times 20$ . (c) Large ducts from the collicular portion of the same specimen showing primary papillae (P) covered with immunoreactive epithelium.  $\times 20$ .

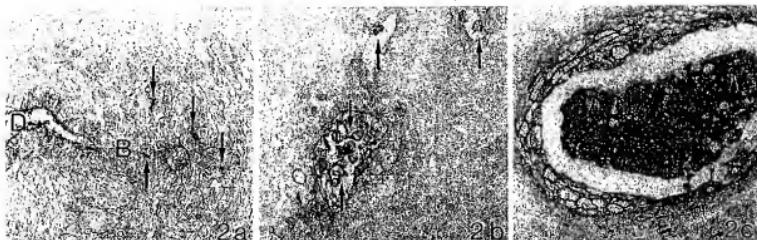


Fig. 2. Incipient lumen formation and focal metaplasia in the prostate of an embryo from the 36th wk of gestation. (a) Spotwise distribution of PMS immunoreactive material (arrows) indicating incipient lumen formation in a solid epithelial prostatic bud (B) originating from a small peripheral duct (D). The adluminal cells of the duct are strongly immunoreactive.  $\times 50$ . (b) Intermediate portion of the same specimen showing desquamating immunoreactive cells (arrows) within the lumen.  $\times 50$ . (c) Periurethral duct with immunoreactive squamous metaplastic epithelium and condensed intraluminal detritus.  $\times 30$ .



Fig. 3. Distribution of PMS immunoreactivity during postnatal development of the prostate. (a) Central portion of the prostate from a 3.5-month-old child showing a group of ducts, some of which contain metaplastic immunoreactive epithelium and newly formed ducts with immunoreactive adluminal cells.  $\times 20$ . (b) Prostate of a 4-yr-old boy with forming lumina (arrows) inside a solid cell mass (B) adjacent to a large duct (D) close to the urethral opening.  $\times 52$ . (c) Maturing prostate from a 16-yr-old boy. Peripheral prostatic acini covered with strongly immunoreactive adluminal cells.  $\times 53$ .

lumen formation renders the multilayered epithelium a cribriform structure (Fig. 3a, b). The basal cells and the neuroendocrine cells never show PMS immunoreactivity on their lateral or basal surfaces.

#### Cell specificity

In prostatic ducts, mostly in their intermediate segments, individual PMS-positive cells start developing both PSA and acid phosphatase immunoreactivity (not shown). Also, the most peripherally located PAS-positive cells (Aumüller et al. 1994) which generally lack PSA or acid phosphatase immunoreactivity, contain an adluminal rim of PMS immunoreactivity (not shown).

Postnatal development of the prostate is most appropriately divided into 3 different phases (Swyer, 1944; Andrews, 1951; Aumüller, 1979): (1) a perinatal

and a subsequent regression phase, (2) an infantile resting period and (3) the pubertal maturation period. During the regression and the resting periods no major deviations from the perinatal situation is recorded with regard to PMS-immunoreactivity (Fig. 3a). During puberty the general development of the ductal epithelium with basal and adluminal secretory cells spreads throughout the gland, starting at about 11–12 y. An increase in the diameter of the lumen of the ducts occurs that is readily recognised from the changing pattern of PMS-immunoreactivity of the developing acini. This is accompanied by the formation of secondary and tertiary ramifications of the glandular ducts, pushing the interstitial stroma aside. At the same time, removal of any remnants of squamous metaplastic epithelium is completed (Fig. 2b), and the number of the PAS-positive cells decreases significantly (not shown).

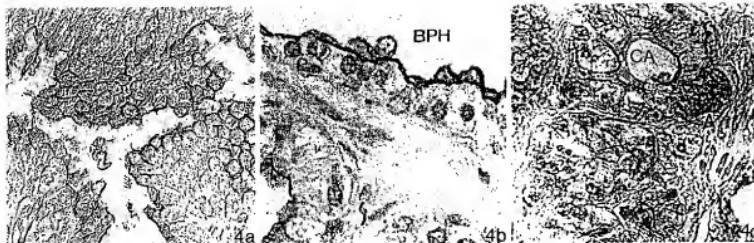


Fig. 4. High power magnification of the labelling distribution in the normal (a, b) and malignant (anaplastic) prostate. (a) Slightly oblique section through apical cell poles (T) showing the labelling of the circumferences of apical plasma membranes.  $\times 200$ . (b) Atrophic epithelium in a case of benign prostatic hyperplasia (BPH) with strongly labelled apical plasma membrane; nuclei counterstained with haemalum.  $\times 750$ . (c) Differentiated prostate cancer with membrane-labelling of gland-like structures (CA). In an adjacent anaplastic portion (A) generalised cytoplasmic labelling is seen; nuclei are slightly counterstained with haemalum.  $\times 600$ .

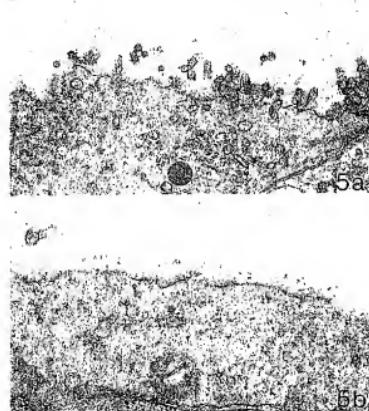


Fig. 5. Immunoelectron microscopy of PMS immunoreactivity in the adult prostate. (a) Immunogold particles are concentrated on the apical plasma membrane of a secretory cell covered by microvilli.  $\times 40000$ . (b) Labelling of the tangentially cut smooth surface of a prostatic secretory cell.  $\times 40000$ .

The formation of mature acini starts at about 15–16 y and can be traced by the changing pattern of PMS immunoreactivity and increasing PSA or acid phosphatase immunoreactivity. Whereas in the large periurethral ducts, which usually contain a multi-layered epithelium until the age of 15–16 y, adluminal cells containing secretory material are interspersed with nonreactive cells, the intermediate duct portion

forms secondary and tertiary ramifications, which gradually distend, forming sacculations separated by papillae (made of connective tissue and smooth muscle cells). The peripheral subcapsular acini are small, round or have few sacculations and their epithelium consists of cells nonreactive for secretory proteins, but have distinct PMS immunoreactivity at their apical plasma membrane.

#### Cell polarity

In the mature prostate the highly purified 100 kDa antibody reacts strongly at the apical plasma membrane (Figs 3c; 4a, b). At the electron microscopic level the exclusive labelling of the outer leaflet of the apical plasma membrane becomes evident (Fig 5a, b). This pattern of immunoreactivity is retained in well differentiated prostate cancer, but lost in anaplastic cancer cells (Fig 4c), where all of the cytoplasm stains or stain is completely lost.

## DISCUSSION

#### Characteristics of the antiserum

In the present study we have used an antiserum raised against prostasomes (Ronquist & Brody, 1985), i.e. particles that were thought to be released from the prostate during ejaculation. Western blotting experiments clearly confirmed the composite variety of antibodies present in the crude antiserum among which, however, one antigen of about 100 kDa was prevalent. Immunoadsorption purification of the relevant antibody (anti-100 kDa) resulted in a highly specific labelling exclusively of the apical plasma membrane of prostatic secretory cells.

The question, therefore, is to which of the known prostatic antigens the 100 kDa antigen would be related and what its nature, prostate specificity and androgen dependence would be. The most likely candidate responsible for the 100 kDa band detected by our immunopurified antibody is the so-called prostate membrane specific antigen (PSM), recently described by Israeli et al. (1993, 1994). Israeli et al. (1994) found the highest PSM expression in hormone-deprived states of the prostate. In benign prostatic hyperplasia, PSM was at times absent. All these features are very much in favour for the assumption that PSM is related to or identical with our 100 kDa antigen present in the apical plasma membrane of prostatic adluminal cells. The respective antibody, therefore, should be well suited for the study of prostatic development.

#### *Organogenesis and maturation of the prostate*

The prostate derives from a composite area of the urogenital sinus (Glenister, 1962) and is thought to require both oestrogens and androgens during organogenesis (Neumann et al. 1974; Pylkänen et al. 1992).

The formal development of the human prostate has been thoroughly studied both at the light and electron microscopic level, e.g. by Kellokumpu-Lehtinen et al. (1980). The 100 kDa antibody used in the present study has the advantage that it easily recognises lumen formation. The immunoreactive adluminal cell layer is the first cell type showing an advancing step of differentiation relative to the basal cells. The 100 kDa antibody, therefore, may serve as a marker of initial prostatic differentiation. A second step of differentiation would then be the androgen-dependent expression of secretory proteins within such cells which are already immunoreactive for the 100 kDa antigen.

Lumen formation is an essential step during glandular organogenesis and is obviously a highly regulated and genetically fixed process which is also retained after malignant transformation. Interestingly, the patterns of lumen formation and development observed during organogenesis are partly reflected in prostate cancer, ranging from the glandular to the cribriform pattern.

Another important aspect in the expression of the 100 kDa antigen is its occurrence during an androgen-deficient state, at least during a period where androgen levels inside the prostate are very low, contrary to the oestrogen levels (Zondek et al. 1986). Whereas the secretory proteins are immunohistochemically

detected only after the onset of puberty (although faint immunoreactivity may be present in the glands of young children), the 100 kDa antigen is present adluminally from the very beginning. This would perhaps indicate that this antigen would be retained in prostate cancer cells, even when they have lost their androgen dependence. The PMS antigen is retained more homogeneously in prostate cancer cells relative to PSA. The only significant deviation from normal cells observed was the increased cytoplasmic staining in anaplastic cells compared with the apical membrane staining in intact cells. In other words, the changed distribution of the antigen in anaplastic cells would indicate a loss of polarity of these cells due to disturbed membrane trafficking.

In conclusion, the prostate membrane specific protein present in human seminal/prostasomal proteins has been detected immunohistochemically on the apical plasma membrane of prostatic adluminal cells. Its unique localisation, already evident during the earliest stages of development, shows that it is synthesised in an androgen-independent manner, which is in contrast to the ordinary secretory proteins of the gland. It may be used as a marker of growth patterns in the normal and pathologically altered prostate.

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